International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.2, No.2, pp 1550-1557, April-June 2010

Design and Evaluation of Drug Loaded Erythrocytes

Sudha S. Rathod[1]*, S. G. Deshpande[1], Dhiraj S. Nikam[2] and Mudra S. Rathod[2]

[1]C. U. Shah College of Pharmacy, Santacruz, SNDT, University, Mumbai, MH,India

[2]V.M.H.P. Shah College of Pharmacy, G.B. Road, Kasarvadavli, Maharashtra, Thane,400 601,MH,India

*Corres. author: sudharathod@yahoo.co.in, sudharathod@gmail.com

Abstract: Encapsulation of a drug into carriers permit delayed or controlled kinetics of release, increased specificity of delivery to target cells or organs and use of novel routes of drug into cells. Erythrocytes are autologous and natural product of the body and are biodegradable as well as nonimmunogenic. The Cells carrying drug can circulate intra-vascularly for prolonged period of time. Hence rat erythrocytes have been loaded with 5-flurouracil (5-Fu), an anti cancer drug, to deliver drug to specific sites in the body. The method used was hypotonic preswelling and isotonic resealing. Drug loaded cells were characterized in vitro for osmotic fragility, drug entrapment, drug and hemoglobin leakage and morphological characteristics. Drug was analyzed by HPLC and HPTLC. Hemoglobin release was measured by UV spectrophotometer. Encapsulation of 65%drug was achieved by this method. Optical microscopic examination of the drug loaded cells revealed no difference in the morphological characteristics of the cells compared to the normal erythrocytes. Cells were discoid in shape. Osmotic fragility test of the loaded erythrocytes and normal erythrocytes were carried out at different sodium chloride concentration viz. 0.1% to 0.9%. Normal erythrocytes released 50% of cellular hemoglobin at the chloride concentration of 0.35% whereas the drug-loaded erythrocytes released the same amount of hemoglobin at 0.5% sodium chloride concentration. Further shelf life of drug-loaded erythrocytes was prolonged by lyophilization. No difference was observed in the shape, size and drug content of loaded and lyophilized erythrocytes when compared with freshly loaded erythrocytes.

Keywords: Resealed erythrocytes, 5-Flurouracil, Encapsulation, Hypotonic-preswelling, Isotonic-resealing.

Introduction and Experimental

Encapsulation of biologically active substances potentially permits the entrapped substances to be protected. *In-vivo* from inactivation or degradation or to be excluded from the immune system. Encapsulation also permits delayed or controlled kinetics of release, increased specificity of delivery to target cells or organs and use of novel routes of entry into cells. Several encapsulation techniques for accomplishing some of these goals have been devised utilizing systems, such as liposomes[1], nanoparticles[2], albumin microsphares[3], magnetic microspheres[4], and erythrocytes[5].Delivery systems, which employ naturally occurring substances as carriers, have certain obvious advantages with respect to biocompatibility. Blood substances have further advantage of rapid and wide spread distribution. Patients own erythrocytes may be used as the carriers, which minimizes complications associated with long-term transfusion.

Three methods are generally used for the loading of compounds into erythrocytes. Osmotic lysis, electric field lysis and dialysis.Figure 1 shows the steps involved in the osmotic lysis and resealing of erythrocytes. In the presence of hypotonic solution, water enters into the cells and cells swell. The cells can swell in size up to 1.6 times the original volume before enough stress develops to rupture the membrane and cause the appearance of pores[6]. These membrane pores are 200-500˚A in size and can allow equilibration of the intracellular and extra cellular volumes. The length of time that these pores remain open is uncertain but at 0˚C they are open long enough to allow partial equilibration of the intra and extra cellular media. Increasing the ionic strength to isotonicity and incubating the cells at 37˚C causes the pores to close and restore the osmotic properties of the erythrocytes[7]. This procedure has been carried out for

the entrapment of enzymes by Zimmerman et al [5] and Updike et al[8].

Encapsulation of 5-Fu in Erythrocytes

5-Fu was encapsulated in erythrocytes by hypotonic preswelling and isotonic resealing procedure described by Field et al [9] with minimum modification. Erythrocyte suspension (2 ml) was centrifuged at 2000 g for 5 min at 4˚C to obtain 1ml of packed cells. To the packed cells, 4ml of 0.65%sodium chloride solution was added and the contents were gently mixed[10-11]. The suspension so obtained was centrifuged at 600 g for 5 min to recover the swollen cells and the supernatant was discarded. A 100 µl of drug solution (mg/ml) was added until the cells reach the point of lysis. Drug solution (300 µl) was found to be sufficient to bring the cells to the point of lysis. Further addition resulted in complete lysis of cells which was observed during the process by optical microscope under oil immersion lens (10 x 100 magnification). Calculated amount of hypertonic saline solution (4ml of 1.1g % Nacl solution / ml packed cells) added to restore the isotonicity. Cells were then washed three times with phosphate buffer solution (PBS) to remove the released haemoglobin and the unentrapped drug. Cells were finally suspended in PBS.

***In Vitro* Characterization of Drug Loaded Erythrocytes**

Morphological Examination

Drug loaded erythrocytes were observed under oil immersion lens using optical microscope. Figure 2 shows the ellipsoidal shape, which did not rupture after loading.

Estimation of 5-Fu from Loaded Erythrocytes

Erythrocytes obtained after three washings were suspended in 25 ml of fresh phosphate buffer (pH 7.4) and was heated at 80˚C for complete rupture of the cells. The suspension was cooled at room temperature and centrifuged at 2500 g for 30 min. Suitable dilutions were made with phosphate buffer and analyzed by HPTLC.

Method development for HPTLC Analysis

For the development of an optimum method, following mobile phases were tried[12] to get the optimum R_f value of 5 FU.

1. Ethyl acetate: Acetone: Water (70: 40: 10)
2. Ethyl acetate: Methanol (75: 25)
3. Ethyl acetate: Methanol: Glacial acetic acid (75: 25: 1)
4. Chloroform: Glacial acetic acid (65: 35)
5. Ethyl acetate
6. Methanol

Out of these solvent mixtures and solvents, ethyl acetate was taken as a mobile phase (R_f= 0.7)

Samples were spotted on silica gel GF 254 (E.Merck) plates (0.25 mn thick) with the help of an automatic sampler and an automated spray technique (Linomat IV applicator). The plate was then developed upto 7cm in a TLC chamber saturated previously with ethyl acetate. Plate was removed from the chamber, dried and scanned in HPTLC scanner CAMAG II N 3.14 model at 266 nm in fluorescence mode. CAMAG software (CATS) was used for peak height and peak area measurement. Figure 3 shows the HPTLC fingerprint of 5-FU standard and Figure 4 shows the fingerprint of 5-FU from erythrocyte. Table 1 shows the linearity of 5-FU standard solution.

***In Vitro* Drug Release**

Three times washed erythrocytes were suspended in the phosphate buffer pH 7.4, maintained at 4˚C in a hard glass tube. Measured amount of fluid from the beaker was removed periodically and replaced by the same amount of fresh fluid. Analysis of drug was carried out by HPLC as described above. Each time standard solution was applied along with sample solution Figure 5.

***In Vitro* Haemoglobin Leakage**

In vitro leakage of haemoglobin from drug loaded erythrocytes were carried out in the same manner as in the *In vitro* drug release. Clear supernatants were withdrawn at regular interval and absorbance was recorded at 540 nm (spectrophotometer Shimadzu UV – 160 A Japan), Figure 5.

Osmotic Fragility Studies

Osmotic fragility studies were performed for normal and drug loaded erythrocytes following the method described by Sprandel and Zollner[13].Erythrocytes (0.5ml packed cells) were incubated in sodium chloride solutions of different strengths (0.9,0.8, 0.7,0.6,0.5,0.4,0.3,0.2 and 0.1g %) for 10 min at 37˚C. The supernatant was withdrawn at regular interval and estimated spectrophotometrically for haemoglobin leakage Figure 6.

***In Vivo* Evaluation of Drug-loaded erythrocytes**

Tissue Distribution Studies

In vivo studies were carried out in albino rats weighing 250 ± 20 gm to find out the survival time of 5-FU loaded erythrocytes. Solution containing (500 µg) 0.5 ml formulation and plain drug solution was given intravenously to a group of 12 rats each. Three rats from each the groups were sacrificed at the end of 1 hr, 3 hr, 6 hr and 24 hr. Various organs such as liver, lungs, spleen and small intestine were isolated, washed

with chloroform, blotted and weighed. All the organs were homogenized in a tissue homogenizer in 1:4 (g/ml) ratio with methanol and centrifuged at 4000 g for 10 min. The supernatant obtained were further diluted and analyzed by HPLC for 5-FU content as follow.

HPLC Method for analysis of 5-FU from tissue samples

Tosoh (PX-8010) HPLC model with a UV detector (Toson UV-8010) operating at 254 nm utilizing a C-18 reverse-phase column (25 cm in length and 4.6 mm internal diameter) was employed. The method was capable of detecting 1μg of the sample and was reproducible under conditions of constant pressure, temperature and pH. Mobile phase used was phosphate buffer containing 2.5% methanol at pH 6.9.Linearity studies were carried out taking 100 ng to 1 μg concentration of 5-FU Table 1.

Stability studies

Stability studies of drug-loaded erythrocytes were carried out in our laboratory on lyophilized product. Product was lyophilized in vials using mannitol a ratio of 1:2 resealed erythrocytes mannitol, using Edwards's lyophilizer. Mannitol was used as a cryoprotectant, which helped in reducing the time required for lyophilization process.Dried powder was stored in an amber color vial at 4˚C for 3 months. Estimation of drug content, shape and release was studied and compared with the freshly prepared drug loaded erythrocytes Table 4. Maximum shelf life reported by Lewis and Alpar[15] was 2 weeks. An attempt was made to obtain the resealed erythrocytes in a powder form by lyophilization, which could be reconstituted in saline before administration and can be stored at 4˚C for one month without alteration in the morphology and in vitro release profile. Kinosita and Tsong [16] demonstrated that erythrocytes carrying ^{14}C- sucrose entrapped by electrical field procedure servived in mice with virtually no alteration of kinetics for 30 days period. Between 78% and 98% of the cells servived depending upon the duration of the electrical pulse.

Results and discussion

A gentle loading method based on hypotonic hemolysis, isotonic resealing and reannealing was employed for the encapsulation of 5-Fluorouracil in erythrocytes**.** Saline solution (0.65%) was used for swelling of the erythrocytes (no visible hemolysis was observed at this concentration). Swollen erythrocytes were brought to the point of lysis by addition of aqueous drug solution. This point was observed microscopically. Various encapsulation parameters such as encapsulation efficiency, in-vitro release of drug and hemoglobin, morphological examination and cell count, osmotic fragility, biological and stability studies were carried out to evaluate the loaded cells. Encapsulation of 68 % of 5-FU, which represented 975 μg/ml of packed erythrocytes was achieved. Optical microscopic examination of the loaded cells revealed no difference in the morphological characteristics when compared with normal cells. The cells were discoid in shape, Figure 2 shows the photograph obtained by optical microscope at 1500 magnification. Fingerprints of 5-FU standard and samples are shown in Figure 3 and Figure 4 respectively. *In-vitro* release profiles of encapsulated 5-FU and hemoglobin from loaded erythrocytes are shown in Figure 5. Drug was slowly released over a period of 24 hr and was analyzed by HPTLC . Osmotic fragility curves of the normal and drug-loaded erythrocytes are shown in Figure 6. Normal erythrocytes released 50% of cellular hemoglobin at the sodium chloride concentration of 0.33 % whereas the drug-loaded cells released the same percentage of hemoglobin at 0.5% salt concentration. Drug loaded erythrocytes released higher concentration of hemoglobin as compared to the normal cells [17]. Hemoglobin loss was found to be about 50% in 24 hr, Figure 6 Erythrocytes have variable survival qualities when reinfused back into experimental animals. Thorpe et al[18] in their study found that the drug loaded erythrocytes were removed rather quickly from the circulation in mice.

To evaluate the *In-vivo* performance of the developed system, tissue distribution of 5-FU following i.v. administration of drug solution and drug loaded erythrocytes of drug was investigated. Drug analysis of tissue samples were carried out by HPLC method[20]. Procedure used for detection of 5-FU from tissue samples was similar as developed by Youcef M. Rustum for quantitative separation of purine and pyrimidine nucleosides and bases [21]. Drug solubility was found in the following order water>methanol>ethanol and was insoluble in chloroform, ether and acetone. Lewis and Alpar [22] achieved similar results by suspending the cells after encapsulation in oxygenated HBSS containing 1% soft bloom gelatin. Table 1. shows the linearity study of 5-FU by HPLC. This method was sensitive enough to detect less than 0.1 μg of the drug (5-FU). Drug was more in the liver after 1 hr and 3 hr studies and decreased in 6 hr and was undectected in 24 hr when free drug was administered i.v. In comparison with the erythrocytic drug carriers, concentration of drug was less in 1 hr but increased in 3 hr and found maximum in 6 hr studies but no drug was not detected after 24 hr Table 2.

Resealed erythrocytes are reported to be cleared from circulation by RES recognition Thorpe et al[18]. Beutler et al[19] infused two patients with human

erythrocytes that had been prepared by dialysis method. In an asplenic patient, the erythrocytes had a ^{51}Cr half-life of 10 days and in second infusion, the erythrocytes displayed a two component survival of 14 hr and 5 days.Concentration of drug in lungs following free drug solution was more in 1 hr and 3 hr. Reduction in drug level was observed in 6 hr studies and found minimum in 24 hr whereas with erythrocytic drug carriers, the concentration of drug found was less in 1 hr and increased in 3 hrs. Reduction in drug concentration was found in 6 hr and 24 hrs studies,Table 3. In spleen when free drug solution was administered, drug was detected in 1 hr 3 hr and 6 hr. No drug was detected in 24 hr studies. With drug loaded erythrocytes, level of drug was slowly increased from 1 hr to 3 hrs and was found maximum in 6 hr, drug level was reduced in 24 hr. Concentration of drug in small intestine was more in 1 hr and reduced in 3 hrs and 6 hr following free drug administration and reduced to considerable extent in 24 hr while with drug loaded erythrocytes, drug level was maintained in 1 hr, 3 hr and 6 hr and went down in 24 hr. Table 5.

From the above observations, it can be concluded that drug loaded red blood cells result in prolonged drug activity which in turn produces safer and more effective therapeutic levels than the conventional dosage forms. It appears that there is substantial protection against degradation of the drug by endogenous proteolytic enzyme or by antibody-complexing due to encapsulation.Further studies based on measurement of metabolite levels in tissues are warranted to strengthen the reasoning of 5-FU as it is reported that the drug is metabolized extensively by variety of routes and the metabolites are effective than the parent drug[23].Administration of developed system resulted in elevated hepatic, splenic, pulmonary as well as intestinal drug concentration which suggest the presence of RES [18]. Few reports are available on the shelf life of drug-loaded erythrocytes. Lewis and Alpar [22] have reported the maximum shelf life for 2 weeks.

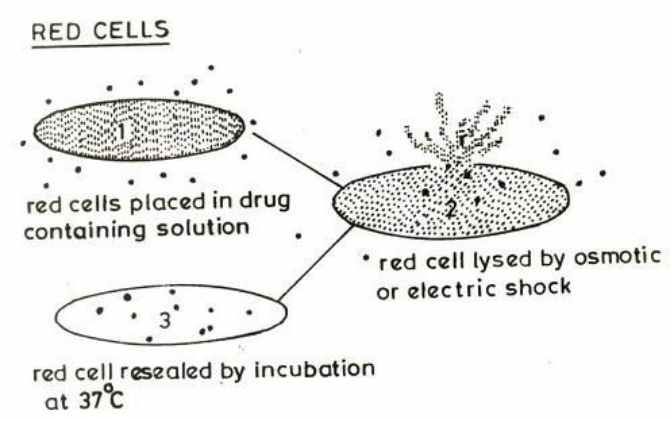

Figure 1. Strategies for preparing erythrocytic drug carriers

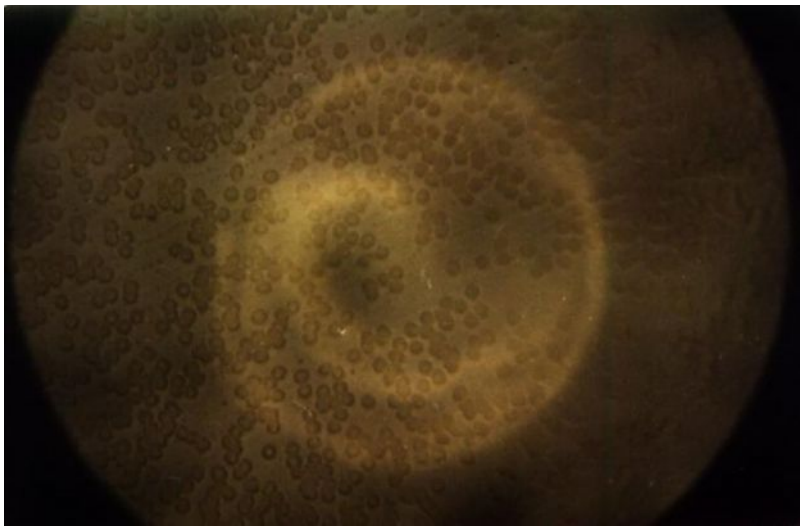

Figure 2. Photograph of drug loaded erythrocytes

Table 1: Linearity Studies of 5-Fluorocil by HPLC

No. of Injections	Area				
	100 ng	**250 ng**	**500 ng**	**750 ng**	**1000 ng**
1	5894	12784	24916	38501	17637
2	5565	13343	25783	39112	50358
3	5210	14637	24636	38913	49885
4	5571	13155	24444	38381	49802
5	4952	13131	25790	38337	47824
6	5199	13151	24140	39045	49759
Mean S.D. ±	5398.5 310.29	13366.83 591.70	24951.66 633.95	38714.8 317.79	49211 1066.2

Table 2: 5-FU Concentration in liver of albino rats after intravenous administration of plain drug and drug loaded Erythrocytes

Time (hrs)	Plain Drug Concentration μg/gm tissue					Drug loaded Erythrocytes Concentration μg/gm tissue				
	1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
1	31.95	30.61	28.88	30.47	1.25	10.63	8.5	12.5	10.54	1.63
3	36.9	37.62	30.8	35.1	3.05	15.7	15.08	16.73	15.83	0.68
6	14.55	15.15	15.97	15.29	0.58	40.35	44.54	38.36	41.07	2.55
24	N.D.	N.D.	N.D.	--	--	N.D.	N.D.	N.D.	--	--

N.D.- Not detected

Table 3: 5-FU Concentration in Lungs of albino rats after intravenous administration of plain drug and drug loaded Erythrocytes

Time (hrs.)	Plain drug Concentration μg/gm tissue					Drug Loaded Erythrocytes Concentration μg/gm tissue				
	1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
1	38.25	36.3	32.5	35.68	2.38	6.3	7.9	4.8	6.33	1.26
3	24.0	23.6	25.88	24.49	0.99	39.6	38.8	38.2	38.86	0.57
6	4.4	5.3	4.9	4.86	0.36	18.63	18.02	17.94	18.16	0.30
24	8.0	8.3	9.7	8.66	0.74	7.07	7.89	6.43	7.13	0.59

Table 4: 5-FU Concentration in spleen of albino rats after intravenous administration of plain drug and drug loaded Erythrocytes

Time (hrs.)	Plain drug Concentration μg/gm tissue					Drug Loaded Erythrocytes Concentration μg/gm tissue				
	1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
1	8.21	6.93	9.96	8.32	1.22	2.82	3.99	3.43	3.41	0.47
3	10.55	13.31	7.44	10.43	2.39	8.25	9.37	7.78	8.46	0.66
6	8.41	5.60	3.33	5.78	2.07	19.3	17.93	18.05	18.42	0.61
24	3.82	3.52	2.8	3.38	0.42	4.14	5.6	3.3	4.34	0.95

Table 5: 5-FU Concentration in small intestine of albino rats after intravenous administration of plain drug and drug loaded Erythrocytes

Time (hrs.)	Plain drug Concentration µg/gm tissues					Drug Loaded Erythrocytes Concentration µg/gm tissues				
	1	2	3	Mean	S.D.	1	2	3	Mean	S.D.
1	13.75	10.2	14.6	12.85	1.89	16.6	15.9	16.8	16.43	0.38
3	6.14	8.4	6.6	7.04	0.97	12.93	13.8	12.2	12.97	0.65
6	6.44	5.3	4.5	5.41	0.79	12.0	13.6	12.8	12.8	0.65
24	1.90	2.01	1.91	1.94	0.04	1.92	1.80	2.01	1.90	0.08

Table 6:Comparative study of freshly prepared and lyophilized drug loaded erythrocytes

Characteristics	Freshly prepared erythrocytes	Lyophilized drug loaded erythrocytes
Shape	Discoid	Discoid
Drug content	68.02%	67.93%
Release on 1st day	90.79%	89.44%
Release on 2nd day	89.49%	88.50%
Release on 4th week	90.23%	88.82%

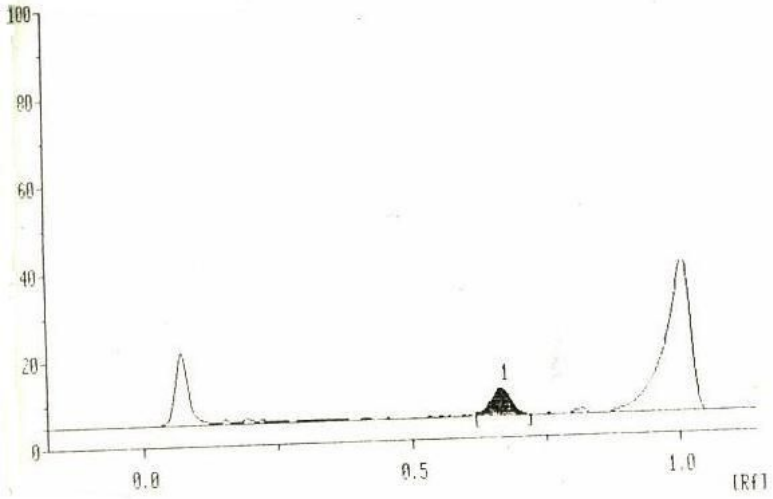

Figure 3. Finger print of 5-FU (Standard) by HPTLC

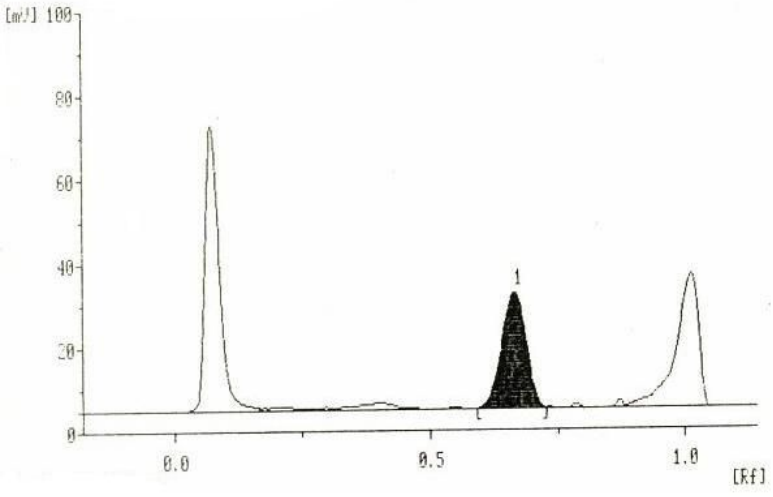

Figure 4. Finger print of 5-FU (Sample) by HPTLC

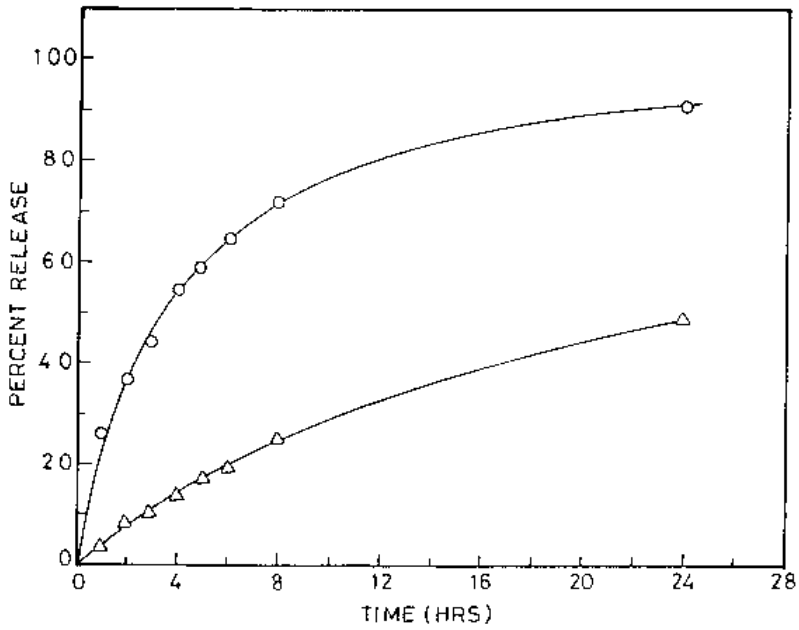

Figure 5. In-vitro release of 5-FU (o) and Haemoglobin (Δ) from drug loaded erythrocytes

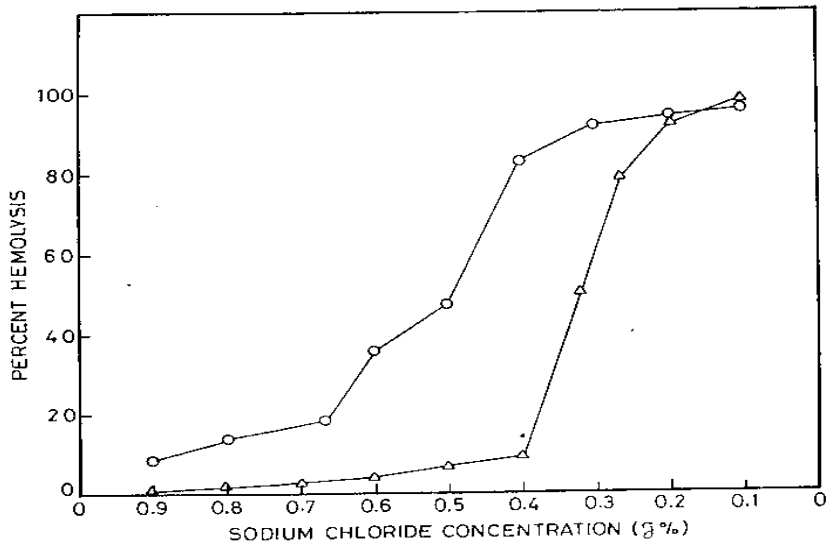

Figure 6. Osmotic fragility curves of normal (o) and drug loaded (Δ) erythrocytes

References

1. Gregoriadis, G.,Comparative effect and fate of non-entrapped and liposome-entrapped neuraminidase injected into rats, Biochem J, 1974, 140,323.
2. Oppenheim, R.C., Nanoparticles in "Drug Delivery System". (Juliano, R.L. ed.), Oxford. Univer. Press, Oxford 1980, 177.
3. Lee, T.K., Composition useful for in vivo delivery of biologics and methods employing same, Science 1981, 213,233.
4. Widder, K., Magnetic Microspheres: Synthesis of a Novel Parenteral Drug Carrier, J. Pharm. sci., 1979, 68,79.
5. Zimmermann, U., Electrical-field induced released of chloroplasts from plant protoplasts, Biochem. Biophys. Acta, 1976, 436,460.
6. Seeman, P.J., Structure of Membrane Holes in Osmotic and Saponin Hemolysis, Cell Biol. 1967, 32,55.
7. Serpersu, E.H.,Increasing field strength or capacitance results in permeabilization to larger

molecules, Biochem. Biophys. Acta, 1985, 812,779.
8. Updike, S.J, Asparaginase entrapped in red blood cells: action and survival, Science, 1976, 193,681
9. Field, W.N., A comparison of the treatment of thyroidectomized rats with free thyroxine and thyroxine encapsulated in erythrocytes, Int. J. Pharm, 1989, 51,175-178.
10. Vyas, S.P.,An erythrocyte based bioadhesive system for nasal route of administratration J Control Release, 1993, 23,231-237
11. Jain,S.K.,Magnetically Responsive DiclofenacSodium- Loaded Erythrocytes: Preparationand In Vitro Characterization, J. Microencapsul. 1994, 11(2),141-151
12. Klause F.,Analytical Profile of Drug Substances, New Jersey, 2,221.
13. Sprandel, V., Osmotic fragility of drug carrier erythrocytes, Res. Exp. Med,1985, 185,77
14. Ihler, G.M., Erythrocyte carriers,Pharmacol. Ther., 1983, 20,151.
15. Lewis, D.A.,In Vitro Characterization of Human Intact Erythrocytes Loaded by Enalarilat ,Int. J. Pharm., 1984,22,137.
16. Kinosita, K., and Tsong, T.Y., Full-length CD4 electroinserted in the erythrocyte membrane as a long lived inhibitor of infection by human immunodeficiency virus, Nature,1978, 272, 258-260
17. Sprandel, V., and Zollner, N., Osmotic fragility of drug carrier erythrocytes, Res. Exp. Med. 1985,185, 77-85
18. Thorpe, S.R., Fidler, M.B. and Desnick, R.J., Enzyme Therapy. V. In Vivo Fate of Erythrocyte-entrapped [beta]-Glucuronidase in [beta]-Glucuronidase-deficient mice Pediatric, John Wiley & Sons Ltd, Res,1989, 9, 918-923
19. Beutler, E., Dale, G.L. and Kuhl, W., Preliminary clinical trial of a new enzyme preparation. (glucocerebrosidase), New Eng. J. Med., 1977,296, 942-943
20. A manual of model TOSOH (PX-8010) HPLC.
21. Youcef, M. R., High-pressure liquid chromatography: I. Quantitative separation of purine and pyrimidine nucleosides and bases, Analytical Biochemistry, 1978, 90, 289-299
22. David, A., Lewis and Alpar, H. O., Control of infectious diseasescatalogue of research projects in the fifth framework programme, Int. J. Pharm. 1984, 22, 1137-1176
23. Therapeutic drug vol. 1, edited by Sir Collin Dollery, Churchill, Livingstone, 1991,68

*****